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ASSIMILATION OF PHOTOSYNTHETIC PRODUCTS OF ZOOXANTHELLAE BY A REEF CORAL[1]

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All hermatypic, or reef building, corals possess autotrophic endosymbiotic algae (zooxanthellae) within their gastrodermal cells. There has been much interest in and controversy about the nature and extent of the contribution of the algae to organic productivity of the coral animal and the reef community (see reviews of Yonge, 1963, and McLaughlin and Zahl, 1966). Ecological studies on some Pacific coral reef communities infer that symbiotic algae in corals are important producers on the reef (Sargent and Austin, 1949, 1954; Odum and Odum, 1955; Kohn and Helfrich, 1957). Measurements of the ratio of photosynthesis to respiration in several coral species from the Pacific and the Caribbean imply that the production of reduced organic carbon by zooxanthellae is sufficient to more than offest losses through respiration (Kanwisher and Wainwright, 1967; Roffman, 1968). This raises the question as to how organic material produced by zooxanthellae might be acquired by the host coral? The most likely alternatives appear to be that algae are digested by the coral; or that algae are released by the coral and returned to it via the predator food chain; and/or that soluble photosynthate may be translocated *in situ* from the algae to the host. We have investigated the latter alternative.

Selective release and translocation of soluble photosynthate by symbiotic algae has been demonstrated experimentally in other coelenterates such as green hydra (Muscatine and Lenhoff, 1963), sea anemones (Muscatine and Hand, 1958; Trench, 1968) and zoanthids (von Holt and von Holt, 1968), in associations of algae and molluscs (Goreau, Goreau, and Yonge, 1965) and in lichens (Smith and Drew, 1965; Drew and Smith, 1967). Goreau and Goreau (1960) attempted to detect translocation of photosynthate from zooxanthellae to host corals by autoradiography. Their conclusion, that little translocation occurred after 50 hours incubation with $^{14}CO_2$, was based on observation of relatively few exposed grains in autoradiographs of sections of coral tissue (minus skeleton). It is is now recognized that the preparation of thin sections removes most of the soluble material from the tissues and the extent of translocation may easily be misinterpreted from autoradiographs, especially if much of the material translocated remains in the host in a soluble form. Von Holt and von Holt (1968) have presented evidence which they interpret as demonstrating translocation of photosynthate in the hermatypic coral *Scolymia lacera*. After incubation with $^{14}CO_2$ for three hours in the light, the pattern of incorporation of ^{14}C among various tissue fractions was noted.

The present paper describes experimental studies on the metabolism of $^{14}CO_2$, the *in situ* translocation of ^{14}C, and its assimilation by the Hawaiian reef coral

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Pocillopora damicornis. Included are data on specific labeled substrates in the algae and animal tissues, the magnitude of heterotropic fixation, the nature of the translocated material, and the acquisition of ^{14}C by the skeleton of *Pocillopora*. This genus of hermatypic coral is common among Indo-Pacific reefs. Intact corals were exposed to $^{14}CO_2$ in the light and dark for varying lengths of time up to 24 hours, and then the amount and nature of the fixed ^{14}C within the coral was ascertained. Of the total ^{14}C fixed photosynthetically by the zooxanthellae, about 35–50% is released and incorporated into host coral constituents.

Methods

Incubation with $Na_2{}^{14}CO_3$ (Step A)

Letters in parentheses refer to steps in the experimental protocol outlined in Figure 1.

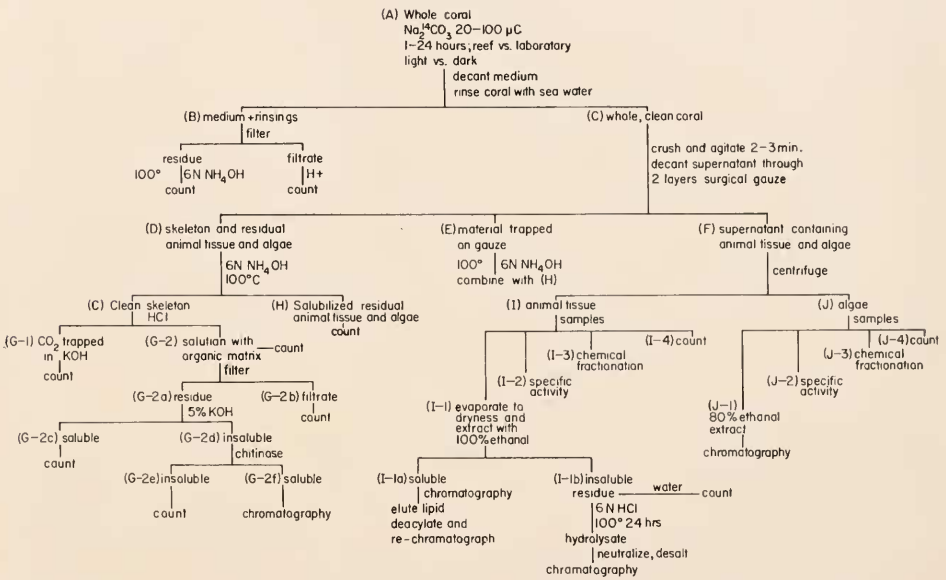

Figure 1. Summary of procedures used to fractionate whole labeled corals.

(*a*) *Laboratory experiments.* *Pocillopora damicornis* was collected from the vicinity of Checker Reef in Kaneohe Bay, Oahu, Hawaii at depths ranging from 1–5 meters. Small heads (30–40 g fresh wt) were dislodged from the substrate by gentle tapping at the base with a geological hammer. Epifaunal organisms were removed and the corals were transported to the laboratory in buckets of sea water. Some corals were maintained at the laboratory submerged in shallow tanks of running sea water pumped from the Bay, and used within a day after collection. Others were immediately placed in a beaker with 200 ml Millipore-filtered sea water to which 25–100 μc $Na_2{}^{14}CO_3$ (1 μc/μl) were added. Beakers were covered with parafilm, placed in a water bath controlled at 25 ± 1.0° C and incubated for 24 hours. The water bath was constructed of ¼ inch plexiglass and placed above

a bank of fluorescent lights delivering 2000 ft-c to the bottom surface of the beaker. Experiments of 1–6 hours were performed only under constant illumination while 24-hour incubations were performed either under constant light or constant darkness. In dark control experiments the isotope was added in a dark room, the beakers were wrapped in aluminum foil and then placed in the water bath.

(*b*) *Reef experiments.* Incubations were carried out *in situ* on the reef for 24 hour periods, starting at 10:00 AM. In initial trials small heads attached to the substrate were enclosed with transparent plastic bags and observed at intervals over 48 hours. Normal underwater surge caused the bags to sway and to chafe the tips of the stationary corals, and bare skeleton was exposed after 24 hours. This method was abandoned in favor of an alternative. Small heads were gently dislodged from the substrate and placed in a plastic bag. The bag was squeezed free of all but about 200 ml sea water and a sealed 10 ml screw cap vial containing 100 μc isotope placed inside the bag. The bag was then tied shut and suspended at the site from which the coral was originally taken, usually at a depth of 3–4 meters. To release the isotope we manipulated the bag so that the vial inside could be grasped and the cap unscrewed, allowing the isotope to mix with the incubating sea water. This incubation method avoided chafing since the bag and coral moved together. The coral, however, was still at its preferred depth and location and at ambient temperature (*ca.* 26° C). The operations were done using SCUBA. After incubation the bag was wrapped in aluminum foil, brought to the surface, and deposited in the laboratory within 20 minutes. After all incubations, either in the laboratory or on the reef, the incubation medium was decanted and the corals rinsed several times with clean sea water. Medium and rinsings (B) were passed through a Whatman No. 1 paper filter. The filtered residue was dissolved in hot 6N ammonium hydroxide, the filtrate was acidified to remove unused carbonate-^{14}C and both fractions were assayed for radioactivity.

Experimental procedures: Fractionation of ^{14}C-labeled corals (*Step C* et seq.)

After incubation, the rinsed, ^{14}C-labeled coral heads were broken into small pieces and placed in an aluminum foil envelope. The broken base of the thick, central branch of each coral head, exposed to the sea water during the incubation, was discarded to avoid error due to labeling by exchange. The remaining coral was crushed with a hammer, quantitatively transferred to a 250 ml flask, and the coarsely fragmented slurry shaken vigorously by hand for 2 minutes. The agitation and abrasive action of the coral chips yielded a brownish supernatant containing algae and animal tissue which was then decanted through several layers of surgical gauze into 12 ml graduated centrifuge tubes. The gauze filter and fragments remaining in the flask were rinsed briefly with clean sea water and the rinsings combined with the supernatant. This treatment yielded two major fractions: a supernatant fraction (F) containing the algae and animal tissue; and a residual fraction (D), left behind in the flask, containing the skeleton and a portion of the algae and animal tissue which could not be freed from the skeleton by the mechanical abrasions. Combined with fraction (D) was the material trapped on the filter (E).

(*a*) *Separation of algae and animal tissue* (*Step F*) Immediately upon obtaining the supernatant mixture of algae and animal tissue, it was centrifuged at 3000 rev/min (International Clinical Centrifuge Model CL). The supernatant sus-

pension of animal tissue was drawn off and saved, and the algal pellet was resuspended in clean sea water and washed twice by centrifugation. The process from abrasion of whole coral to separated algal and animal components took about 5 minutes. Rapid separation is essential since the algae can be expected to release organic material when suspended in the homogenized animal tissue (Muscatine, 1967) (See Discussion). The washings were combined with the animal tissue fraction (I) which was adjusted to known volume and counted (I-4). The algae (J) were suspended in hot 80% alcohol, adjusted to known volume and assayed for radioactivity (J-4). The total activity of the separated algal and animal fractions from each experiment was used in the calculation of per cent translocation in that experiment.

To aid in the estimation of efficiency of separation of algae and animal tissue, mouse liver protein, labeled with ^{35}S as described by Lenhoff (1961), was employed as a tracer (See Results).

(*b*) *Chemical fractionation of tissue* (*Steps J-3, I-3*) Samples of coral tissue and algae were each fractionated by differential solubilities using the method of Roberts, Abelson, Cowie, Bolton and Britten (1955) as modified by Lenhoff (1961). Four major fractions were obtained by sequential treatment with cold 5% trichloroacetic acid (Cold-TCA); acidified 80% ethanol (Alc-sol); and hot 5% TCA. The final fraction was the material insoluble in TCA and alcohol (Alc-TCA-Insol.). This fractionation procedure was used only for quantitative estimation of the fate of labeled carbon in the algae and animal tissues. For qualitative analysis, animal tissue, freshly separated from the algae, was evaporated to dryness and extracted twice with 10 ml hot dry absolute ethanol (I-1). The virtually salt-free ethanol-soluble extract was processed for radiochromatographic analysis (I-1a). The alcohol-insoluble residue (I-1b) was resuspended in water to dissolve salt, was centrifuged and the aqueous supernatant assayed for a radioactivity and then discarded. The remaining salt-free alcohol-water insoluble residue was hydrolyzed with 6 N HCl at 106° C for 24 hours in a sealed tube. The resulting hydrolysate was diluted with several volumes of distilled water, evaporated to dryness, taken up in absolute ethanol and saved for radiochromatographic analysis. The algae were extracted (J-1) and analyzed in an identical fashion except that insoluble material was not hydrolyzed.

Experimental procedures: Assay of skeletal $^{14}CO_3^=$ *and organic matrix-*^{14}C (*Step D* et. seq.)

(*a*) *Assay of residual animal tissue and algae* Skeletal fragments comprising the residual fraction (C) were boiled in 6 N NH_4OH to dissolve and remove residual animal tissue and algae. The tissue-free skeleton (G) was washed in distilled water. The washings were combined with the dissolved organic material (H), and an aliquot of this material was counted. From the radioactivity in fractions (H), (E), (J-4), and (I-4) the total radioactivity in the coral tissues (residual tissues plus separated animal tissue and algae) in each experiment was ascertained.

(*b*) *Assay of skeletal* $^{14}CO_3^=$ Clean skeleton (G) was dissolved in cold 6 N HCl in an evacuated flask (see Doty and Oguri, 1959) and the carbon dioxide collected in 8 N KOH. The KOH fraction containing the $^{14}CO_2$ (G-1) was counted.

(*c*) *Analysis of the skeletal organic fraction* The HCl fraction containing the skeletal organic material (G-2) was counted. The insoluble portion was collected by filtration and washed several times with distilled water. Filtrate and washings were assayed for radioactivity (G-2b).

The washed insoluble material (G-2a) was assumed to be chitin, the chief constituent of the matrix in *P. damicornis* (Wainwright, 1963). This material was suspended in 4 volumes of 5% (w/v) KOH and boiled to dissolve nonchitinous organic constituents (Reynolds, 1954). Soluble material (G-2c) was counted. The insoluble chitin (G-2d) was washed until the effluent was neutral, solubilized and re-precipitated, and incubated with chitinase (Calbiochem #220471) according to the method of Skujins, Potgeiter and Alexander (1965). The reaction was stopped by boiling. After centrifugation, the supernatant (G-2f) was taken to dryness under reduced pressure, then taken up in hot 80% ethanol and co-chromatographed with authentic glucosamine and N-acetyl glucosamine. The insoluble material remaining after chitinase hydrolysis (G-2e) was counted.

Analytical procedures: Total protein

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin standards (Armour and Co.).

Analytical procedures: Radiochromatography and assay of radioactivity

All tissue fractions which were assayed for radioactivity are indicated in Figure 1. Aliquots of known volumes of material were assayed by conventional planchet and liquid scintillation techniques with corrections for background. Two-dimensional chromatography was carried out on soluble samples using Whatman no. 4 paper and phenol : water (100 : 39, w/v) in the first dimension and butanol : propionic acid : water (1246 : 620 : 884 v/v/v) in the second dimension. Radioactive compounds were located on chromatograms with Kodak Blue-sensitive medical x-ray film as described by Bassham and Calvin (1962).

Analytic procedures: Lipid deacylation (I-la)

Ethanol-soluble lipids from animal tissues were eluted from paper chromatograms with a mixture of absolute ethanol and 10% (v/v) toluene. An aliquot was counted and the remainder was dried under nitrogen. To the residue, 100 μl 0.2 *M* KOH in dry methanol (equal volumes) was added. The suspension was incubated at 35° C for 30 minutes and then water added until a white precipitate appeared. Nine mg Dowex 50 H^+ (200–400 mesh) was added with shaking followed by 1 ml diethyl-ether. Centrifugation yielded a biphasic suspension. The upper layer containing the nondeacylated material was counted and discarded. The bottom layer was taken up in 0.5 ml of the phenol : water solvent and chromatographed.

Analytical procedures: Identification of unknowns

Unknown radioactive compounds were provisionally identified by cochromatography as described previously (Muscatine, 1965, 1967).

Results

^{14}C fixation by intact corals in light and dark

Table I shows that intact corals fixed $^{14}CO_2$ in the light and in the dark. Over a 24 hour period the amount of fixation by corals on the reef was significantly higher than in the dark controls. Fixation in the dark was about 10–12% of that in the light. Evidence given below and in Table I shows most of the ^{14}C fixed in the light over a 24 hour period accumulated in the coral tissues and only 9–12% in the skeleton. It is concluded that in the light, the bulk of the ^{14}C fixed in the living tissues was the result of photosynthesis by the zooxanthellae, and that a smaller proportion was fixed heterotrophically.

Table I

Distribution of total ^{14}C in major constituents of P. damicornis *in three 24-hour incubations*

	Reef-24 hours				Dark-24 hours	
	cpm	%	cpm	%	cpm	%
Medium	92,000	0.9	58,000	0.7	4,500	0.6
Animal tissue*	4,349,000	45.0	2,463,270	32.0	200,889	25.7
Algae*	4,268,000	44.2	4,281,770	55.5	203,186	26.1
Skeleton**	(28.2g)		(26.9g)		(29.1g)	
As carbonate†	927,000	9.6	884,930	11.5	336,150	47.0
As matrix†	17,065	0.2	16,220	0.2	4,147	0.5

Corals of approximately the same dry skeletal weight were incubated in equal volumes of sea water containing 100μC $Na_2\,^{14}CO_3$.

* These totals include radioactivity in the residual tissue which was assayed and added to the already separated animal tissue and algae in their respective proportions. Proportion of radioactivity in animal tissue given in Table IV, data for 24 hours.

** Total skeletal ^{14}C was determined from the product of skeletal weight (in parentheses) and the average specific activity (cpm/g skeleton). The average specific activity was calculated from data for 24-hour incubations in Table VI.

† The proportion of ^{14}C as carbonate is the difference between the total skeletal ^{14}C and the matrix ^{14}C. Matrix ^{14}C was calculated from the data for average percent of ^{14}C in the matrix in 24-hour incubations in Table VI.

In all experiments with intact corals less than 1.0% of the total fixed ^{14}C was detected in the incubation medium. This exogenous organic radiocarbon may have originated either from labeled coral mucus, from bacteria residing on the surface of the coral, from excreted soluble metabolites, or from discharged zooxanthellae.

Removal of coral tissue from skeleton

To separate the algae and animal tissue, the tissues first had to be removed from the skeleton. Mechanical abrasion as described under Methods removed most of the living tissues but some tissue was invariably retained in the residual fraction (D). Table II shows that of the total labeled coral tissue in each of 18 experiments a mean value of $52 \pm 11\%$ was obtained for analysis. There are no

TABLE II

Recovery of ^{14}C-labeled tissue (animal plus algae) from P. damicornis *homogenates*

Incubation time (hours)	Recovery (%)
Laboratory (light)	
1	30,47
2	60,46
3	47,39
4	53,53
5	50,55
6	43,48
24	63,70
Reef (ambient light)	
24	81,37
Laboratory (dark)	
24	54
	$\bar{X} \pm$ S.D. = 52 ± 11

The total labeled tissue was the sum of the recovered tissue (F) plus the residual tissue (D plus E). Per cent recovery was then calculated as (recovered/recovered + residual) × 100.

obvious or significant correlations between amount of tissue recovered and incubation conditions.

Efficiency of separation of algae and animal tissue

Examination of suspensions of algae separated from animal tissues by centrifugation revealed traces of non-algal debris such as nematocycts, *etc.* Occasionally, algal cells could be found in the animal tissue fraction. To estimate accurately translocation of ^{14}C from algae to animal it was essential first to measure the extent of mutual contamination of each fraction.

To determine the extent to which the animal fraction contaminated the algae, finely homogenized ^{35}S-labeled mouse liver was added to an unlabeled coral homogenate to serve as a tracer for "animal tissue." After centrifugation, in three trials, 6.1, 6.8 and 5.0 per cent of the labeled liver added was recovered in the algal pellet. To determine the extent to which the algae contaminated the animal fraction we had planned to add a suspension of labeled zooxanthellae to a "cold" homogenate, centrifuge to pellet the algae and then assay the supernatant for radioactivity. But since coral homogenate stimulates excretion by the algae (Muscatine, 1967), this approach had to be modified by adding the labeled algae to boiled homogenate, in which the stimulatory activity is destroyed. In two trials, 0.5 and 2.0 per cent of the total activity added was recovered in the supernatant. From these data we conclude that the separation of algae and animal tissue from *P. damicornis,* as described here, is about 95% efficient and mutual contamination of fractions does not constitute significant error, either in the estimation of translocation or in the qualitative analysis of the fractions.

Proportion of animal to algal tissue

To estimate accurately the magnitude of translocation it was important to determine the variation in the relative amounts of algae and animal tissue in representative samples. Table III shows that ratios of animal to algae protein ranged from 0.61 to 1.59. This wide range was not entirely unexpected since removal of tissue from the skeleton was incomplete, and numbers of algae per unit coral tissue vary among colonies of *P. damicornis* and other coral species (our unpublished observation; Goreau, 1963). The interpretation of this variation and its treatment in estimating translocation is given below.

TABLE III

Relative amounts of animal and algae protein in samples recovered from whole coral

Coral sample wet weight (grams)	Animal fraction mg protein/sample	Algal fraction mg protein/sample	Animal protein/ Algal protein
31.5	3.16	2.68	1.18
27.0	3.26	2.81	1.16
28.5	2.11	2.11	1.00
27.0	3.26	2.05	1.59
31.0	2.11	3.07	0.69
25.0	2.11	3.07	0.69

Photosynthesis and release of photosynthate by algae in vitro

It has already been shown that zooxanthellae isolated from *P. damicornis* can release up to 40% of their photosynthate when in the presence of homogenate of their host tissues (Muscatine, 1967). These preliminary studies were confirmed here. Figure 2 and 3 are, respectively, autoradiographs of chromatograms of the medium and of an 80% ethanol-soluble cell extract of zooxanthellae incubated in the light for 2 hours with host homogenate and $Na_2{}^{14}CO_3$. Figure 2 shows the major products released by the cells. The relative proportions are glycerol, 82%; glycolic acid, 6.4%; alanine, 1.7%; glucose, 0.7%; unknown, 8.9%. The very striking difference in chromatographic pattern of ^{14}C in the medium and in the cell extract (Figure 3) illustrates that glycerol is released by a selective process and not by cell lysis. If the cells had lysed, a wide range of water soluble intracellular compounds would appear in the medium. As noted in the preliminary study, the relative proportions of material released to the medium may vary, especially in incubations of more than 2 hours. In such incubations glycolic acid and other organic acids are detected in greater abundance, and in the presence of host homogenate, the excreted glycerol may be incorporated into lipid *in vitro*. The host homogenate alone neither manufactures ^{14}C-glycerol nor fixes appreciable amounts of $^{14}CO_2$. Finally, the pattern of photosynthetic products of the algae *immediately after isolation* as shown in Figure 3 is virtually identical with those of algae labeled *in vitro* (Fig. 1, fraction J-1). Identification of the labeled intracellular constituents was limited to glucose, the major carbohydrate, alanine and glycerol. Intracellular ^{14}C-glycerol in Figure 3 is 6.6% of the total soluble ^{14}C detected within the cells.

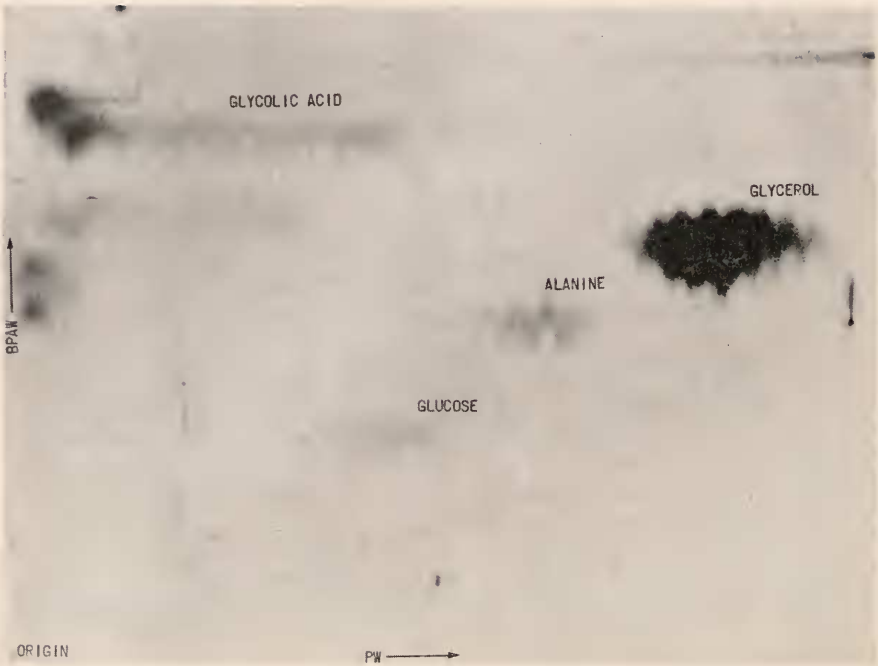

FIGURE 2. Radioautograph of chromatogram of medium from zooxanthellae incubated in light with $Na_2{}^{14}CO_3$, for 2 hours, showing labeled soluble extracellular products.

Translocation of fixed ^{14}C from algae to animal tissue in vitro

Translocation has been measured in other associations as that per cent of the total ^{14}C fixed which is detected in the heterotroph. Measurement of translocation in reef corals is not as straightforward since the skeleton also acquires ^{14}C in the carbonate and in the organic matrix. Data from light and dark experiments in Table IV suggest that some of the activity in the skeletal components could be acquired through fixation by the algae and translocation, and some by direct assimilation of $^{14}CO_2$ from the environment. To simplify our interpretation, and until the source of ^{14}C in the skeleton can be defined critically, we express translocation as that fraction of the total ^{14}C in the separated tissues which is detected in the animal tissue only, omitting from consideration the skeletal ^{14}C. This parameter is distinct from per cent distribution of ^{14}C as expressed in Table I. The amount of ^{14}C in animal tissue as a function of incubation time is shown in Table IV. Even after one hour incubation appreciable ^{14}C is detected in the animal tissue. The precentage rises to a maximum of almost 42% after 24 hours in laboratory incubations (constant illumination) and 36–50% in the animal tissues of corals incubated for 24 hours on the reef.

The appearance of such relatively large amounts of ^{14}C in the animal tissue is taken as evidence for translocation of ^{14}C from algae to animal *in vivo*. Comparison of relative tissue specific activities (cpm/μg protein) in Table IV gives an alternative estimate of translocation, and one which is independent of variation in

TABLE IV

Estimates of in vivo *translocation of* ^{14}C

Incubation time (hours)	Radioactivity in animal tissue (%)	Specific activity (cpm/μg protein)	
		Animal	Algae
Laboratory (light)			
1	14.6	86	361
2	19.5	296	681
3	23.2	541	1180
4	26.9	533	1451
5	34.5	1573	1624
6	34.7	1218	939
24	41.8	*	*
Reef (ambient light)			
24	50.3, 36.5	*	*
Laboratory (dark)			
24	49.7	27	116

Radioactivity in animal tissue was estimated as a percentage by (cpm animal/cpm animal + algae) × 100. Asterisk denotes data not obtained.

sample mass observed in Table III. However, the specific activity determinations assume that algae and animal tissues each have a relatively constant protein content per unit volume. We have not verified this experimentally for *Pocillopora*. The specific activity data indicate that the amount of ^{14}C increases steadily in both tissues through five hours of incubation. A slight decrease in activity was observed in the 6-hour samples. About 50–55% of the fixed $^{14}C/\mu g$ protein was detected in the animal tissue after this time. This value compares favorably with the 50% level of excretion exhibited by freshly isolated zooxanthellae (Muscatine, 1967).

Compared to corals incubated in light, the dark controls in Table IV exhibit relatively low specific activity after 24 hours, confirming that only a small fraction of the ^{14}C in the animal tissue of illuminated corals is due to heterotrophic fixation, and implying that in the light most of the fixed ^{14}C in the tissues is the result of algal photosynthesis and translocation. This interpretation also assumes that in the light the algae do not stimulate heterotrophic fixation by the animal tissue.

Distribution of ^{14}C *in coral tissues in light experiments*

(*a*) *Qualitative analysis* (*I-1, I-1*) From 27 to 43% of the total animal tissue-^{14}C from 24-hour incubation was ethanol-soluble (I-1a). Analysis by paper chromatography revealed a large labeled lipid component and a variety of other labeled compounds, probably low molecular weight substances referable to the TCA-soluble pool (see below). In view of the role of glycerol in lipid synthesis and its involvement in translocation, it was important to ascertain which component of the animal tissue lipids were ^{14}C-labeled. After the lipid was eluted, deacylated and rechromatographed, two radioactive spots were invariably detected. One was judged to be a portion of the original lipid which was not deacylated; the

FIGURE 3. Radioautograph of chromatogram of 80% ethanol soluble extract of zooxanthellae treated as in Figure 2, showing labeled intracellular products.

other was identified as glycerol-^{14}C. No other labeled components were detected. Since most of the ^{14}C released by freshly isolated zooxanthellae is glycerol, we conclude that some glycerol-^{14}C is released *in vivo* by the zooxanthellae and then esterified with unlabeled animal compounds to form lipid.

From 56 to 73% of the animal tissue-^{14}C was ethanol insoluble (I-1b). Radiochromatographic fingerprinting of an hydrolysate of this material revealed that nearly all of the ^{14}C was ninhydrin-positive and referable to amino acids. Two compounds did not react with ninhydrin and behaved chromatographically like native and oxidized lipid, despite treatment of the insoluble fraction with hot alcohol. We conclude that the labelled ethanol-insoluble fraction of animal tissue is largely protein.

(*a*) *Quantitative analysis* (*I-3, J-3*) Table V shows the percentage distribution of ^{14}C in differentially soluble fractions of animal tissue and algae from three 24-hour reef incubations. In both tissues, the relative proportions of labeled cold TCA-soluble (amino acids, sugars, and other low molecular weight substances released by osmotic shock) and hot TCA-soluble (nucleic acids) fractions are roughly the same. It is the alcohol-soluble and alcohol-TCA-insoluble fractions which differ. In the animal tissue more than half of the activity is insoluble protein. About 20% is alcohol soluble. The reverse seems to hold for the algae,

TABLE V

Per cent distribution of radioactivity in differentially soluble fractions of animal tissue and algae after incubation for 24 hours on the reef; data from three different experiments

Fractions	Animal tissue			Algae		
	a	b	c	a	b	c
Cold TCA-soluble	19.3	19.0	18.2	16.3	14.6	18.4
Alcohol soluble	19.3	19.3	22.6	42.7	48.1	42.3
Hot TCA-soluble	4.0	3.0	3.1	6.4	12.4	15.3
Alcohol-TCA-insoluble	57.3	58.5	56.0	34.4	24.8	23.9
% recovery	89.3	91.2	94.1	76.7	86.7	98.7

with the alcohol-soluble fraction comprising the largest proportion of activity and the alcohol-TCA-insoluble fraction ranging from 25–35%. There is thus an apparent reciprocal relationship between the proportion of soluble and insoluble labeled fractions in the algae and animal tissue after 24 hours.

Distribution of ^{14}C in coral tissue from dark incubations

Table I shows the extent of ^{14}C fixation after 24 hours in the dark. Data for dark incubations in Table IV implies that both the animal tissue and the algae can acquire ^{14}C by heterotrophic fixation, or that there is some exchange of ^{14}C-labeled products of dark fixation between animal tissue and algae. The relative extent of each of these processes is not yet known. Only the animal tissue fraction was analyzed qualitatively. Labeled carbon was detected predominantly in the 100% ethanol-insoluble fraction. The radiochromatographic pattern of distribution of ^{14}C in this fraction (I-1b) after hydrolysis was qualitatively similar to the analogous animal tissue fraction in light incubations. The same ninhydrin-positive compounds referable to amino acids were detected. From this we conclude that heterotrophic fixation by *Pocillopora* contributes ^{14}C to proteins, and that this contribution is significantly augmented in the light by photosynthesis by the zooxanthellae. Due to the low activity of the soluble fraction of the animal tissue incubated in darkness, it was not analyzed. It would be interesting to know the extent to which the lipid in this fraction is ^{14}C-labeled.

^{14}C in the skeleton

Table I shows that on a percent basis, the skeleton of corals incubated on the reef for 24 hours contained about 10–12% of the total ^{14}C in the coral, while skeleton from 24-hour dark incubations accumulated 47% of the total ^{14}C. However, illuminated corals acquired 2–3 times as much ^{14}C in both the skeletal carbonate and matrix fractions as coral incubated in darkness. Table VI shows the results of analyses of small samples of cleaned skeleton after various incubation conditions. These data are based on total ^{14}C in skeleton and matrix only.

TABLE VI

Distribution of ^{14}C in skeletal carbonate and skeletal organic matrix in samples of cleaned skeleton

Incubation time (hrs)	Weight of skeleton (mg)	Total ^{14}C (cpm)	Per cent ^{14}C as $^{14}CO_3^=$	Per cent ^{14}C as organic material*
Laboratory (light)				
1	826	12,149	94.3	5.7
2	806	18,550	93.1	6.9
3	809	13,045	95.4	6.6
4	1002	33,141	95.9	6.1
24	811	23,270	96.2	7.8
Reef (ambient light)				
24	809	27,604	98.2	1.8
	806	35,444	98.8	1.2
	805	18,240	97.9	2.1
Laboratory (dark)				
24	807	7,917	99.2	0.8
	810	14,702	99.0	1.0
	803	6,495	98.6	1.4
	828	8,772	98.7	1.3

* Denotes total labeled organic material of which less than 50% may be chitin. See text for complete explanation.

Coral skeleton exhibited appreciable radioactivity after all of the tissue had been removed with alkali. Skeletons of those corals incubated in light in the laboratory for 1–4 hours or for 24 hours contained 93–96% of their ^{14}C as carbonate with specific activities between 14 and 33 cpm/mg skeleton. About 6% of the ^{14}C was acid stable and assumed to represent constituents of the organic matrix. In 24-hour reef experiments, about 98% of the activity in the skeleton was present as carbonate-^{14}C (sp. act. 22–44 cpm/mg) and only 1.2–2.1% was acid stable skeletal organic material. This slightly lower percentage of ^{14}C in the matrix in 24-hour reef experiments may result from the reef incubation which included a dark period, in contrast to the constant illumination in laboratory experiments. Even so, the absolute amount of ^{14}C in the matrix from reef incubations was still higher than that in the dark. The lowest level of incorporation of ^{14}C into skeletal carbonate (sp. act. 9–18 cpm/mg) and into organic material (1.1%) was observed in 24-hour dark incubations in the laboratory. In the dark, it is likely that ^{14}C in the organic matrix originates from heterotrophic fixation and incorporation into newly synthesized matrix. If so, then subtraction of the dark values from the light values may give a valid estimate of matrix labeling as a result of translocation of ^{14}C from the algae.

To determine, in a preliminary way, which matrix constituents were labeled, samples of skeleton from incubations in light were dissolved in cold 6 N HCl and the insoluble material remaining was collected and washed in distilled water; the washings contained negligible radioactivity. This insoluble skeletal organic material was treated and assayed as given in Figure 4. Boiling in hot KOH removed non-chitinous constitutents but the alkali treatment also dissolved about 60% of the radioactivity in the insoluble starting material. Although this alkali-soluble

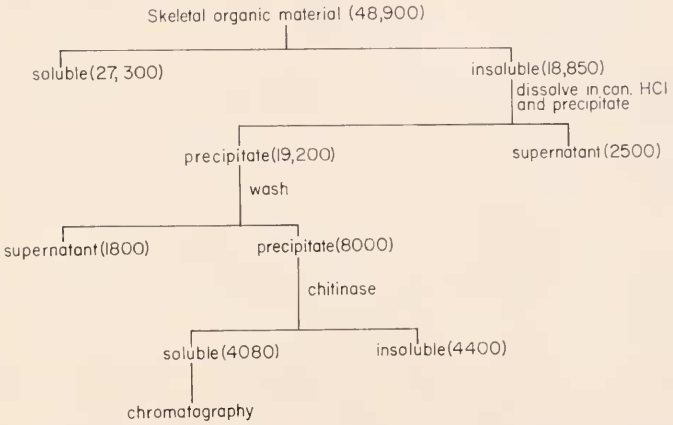

FIGURE 4. A representative flow diagram with quantitative data on analysis of matrix-^{14}C from *Pocillopora*. Numbers in parenthesis are total counts min^{-1} in the sample.

fraction was not analyzed its high activity suggests that either the matrix was contaminated with labeled coral tissue or that other constituents of the native matrix (see Discussion) were labeled and possessed high specific activity. The alkali-insoluble material, presumed to be chitin (or possibly chitosan), was hydrolyzed in a range of HCl concentrations at 100° C for times varying from 1–24 hours in a sealed tube. Under these conditions, nearly all of the activity was invariably lost from the hydrolysate. To explain this we reasoned that much of the activity in the matrix may have been associated with the acetyl moiety of the chitin polymer, and under acid conditions was liberated as acetic acid (Kent and Whitehouse, 1955). Alternatively, treatment of the alkali-insoluble material with chitinase yielded a soluble labeled fraction representing only 10% of the original label in the starting material. Two soluble labeled products were isolated by chromatography. Cochromatography with chitobiose and N-acetylglucosamine proved inconclusive and the products remain as yet unidentified. Matrix from dark incubations has not yet been analyzed.

DISCUSSION

The results presented here show how zooxanthellae contribute to organic productivity of a reef coral. In the light, intact corals fix considerably more $^{14}CO_2$ than those kept in the dark, due principally to photosynthesis by the zooxanthellae. As zooxanthellae fix $^{14}CO_2$ in the light, there is a progressive increase in ^{14}C content of the animal tissue until, after 24 hours, some 32–45% of the total ^{14}C in the coral is present in the animal tissue. Since freshly isolated zooxanthellae excrete photosynthate in substantial amounts in the presence of host tissue homogenate, and since the proportion of fixed ^{14}C excreted *in vitro* (up to 50%) corresponds approximately to the proportion of fixed ^{14}C accumulated by the animal tissue *in vivo*, we conclude that in the intact reef coral *Pocillopora damicornis*, zooxanthellae release a substantial proportion of their photosynthate to the animal

tissue. Von Holt and von Holt (1968) reported translocation values of 40% in the coral *Scolymia* after 3 hour incubations.

The only alternative way that ^{14}C could appear in the animal tissue is through heterotrophic fixation in the light at a rate comparable to photosynthetic fixation. There is no evidence that this is the case. On the contrary, heterotrophic fixation by corals in darkness was at most 10–12% of photosynthetic fixation in the light.

The detection and measurement of translocation depends to a large extent on the efficiency of separation of animal tissue from algae. Two independent measurements used here show that the separation of *Pocillopora* constituents is relatively efficient. This is an important parameter in the analysis of translocation since relatively few algae with high specific activity could introduce significant error in analysis of animal tissue. Von Holt and von Holt (1968) reported removal of tissue from *Scolymia lacera* by "aspiration" but neither the details of the technique nor the efficiency of recovery nor the efficiency of separation of algae and animal tissues was reported. During the separation procedure host tissue homogenate continues to stimulate excretion of glycerol (Muscatine, 1967), but to no greater extent than in the intact association. Consequently some 5 minutes of *in vitro* excretion of glycerol is incorporated into each translocation experiment. If a correction for heterotrophic fixation is applied, the estimate of 35–50% translocation in a 24 hour experiment on the reef, despite its agreement with *in vitro* excretion, may actually be closer to 25–40%. This value still represents a respectable export of photosynthate by the algae, especially since apparently most free-living algae which have been studied rarely release more than 5% of their soluble photosynthate over a 24 hour test period (see Hellebust, 1965).

While we have not yet observed directly the form in which carbon is translocated in the intact coral, we suggest that some glycerol or its derivative is released by the algae and then incorporated in part into animal tissue lipid since ^{14}C-glycerol was detected in the animal tissue lipid. Release and incorporation of glycerol into lipid also occurs in *in vitro* incubations with host homogenate. But lipid is not the only animal constituent which acquires the ^{14}C label. More than half of the ^{14}C in the animal tissue was detected in the protein fraction (alcohol-TCA-insoluble). To account for this, we speculate that either some of the glycerol is metabolized to amino acids, or that other products are translocated in the intact coral but are not excreted in abundance by freshly isolated zooxanthellae. After long *in vitro* incubations with host homogenate zooxanthellae release glycolic acid, and traces of alanine and glucose (Muscatine 1967), suggesting that these might be *in vivo* translocation products. However, experiments performed by Trench (1968) show that there is a gradual change in the pattern of excretion by zooxanthellae from a variety of coelenterates after they have been removed from the host. At first, glycerol is the major constituent to be released, but three hours or more after isolation, other labeled compounds appear in greater relative proportion and glycerol excretion declines. A similar phenomenon has been observed in the lichen algae after they are separated from their fungal associates. In this case there is direct evidence that the compound moving between the algae and host fungi *in vivo* is the same as that excreted by freshly isolated algae, and that the additional

compounds excreted by algae after longer *in vitro* incubations do not move *in vivo* (Smith, Muscatine and Lewis, 1969).

The observation that glucose is the major intracellular product of these zooxanthellae while a different compound, glycerol, is the major extracellular product, is consistent with observations of a similar nature in other symbiotic associations. For example, zoochlorellae from hydra and *Paramecium bursaria* manufacture and retain sucrose as their major intracellular product, but release carbohydrate as maltose (Muscatine, Karakashian, and Karakashian, 1967).

The reciprocal relationship between soluble and insoluble compounds in *Pocillopora* algae and animal tissue may be significant. Smith, Muscatine, and Lewis (1969) have discussed the maintenance by symbiotic algae of a large reserve of soluble photosynthate available for translocation. The conversion of these compounds by the host into insoluble derivatives may promote a one-way movement of photosynthate. Whereas we find that less than 50% of the ^{14}C in the animal tissue is present as soluble compounds (20% as lipid) after 24 hours, von Holt and von Holt (1968) report more than 80% of the ^{14}C in *Scolymia* animal tissue as soluble compounds (50% as lipid) after three hours incubation. We speculate that this difference is a reflection of the metabolism of different coral species, and especially different incubation times. This also serves to emphasize that the preponderance of soluble material in coral animal tissue after short incubations with $^{14}CO_2$ precludes the use of autoradiography to measure translocation. Since our incubations were much longer, our pattern of distribution of ^{14}C in animal tissue may more nearly approach a "steady state" distribution.

The skeletal matrix of *P. damicornis* consists primarily of chitin (Wainwright, 1963). The fact that a portion of this matrix is hydrolyzed with chitinase is consistent with this observation. Further studies will be required to explain the presence of the large amount of labeled alkali-soluble material associated with the decalcified skeleton. In view of their low productivity (Kanwisher and Wainwright, 1967; Halldal, 1968) it is unlikely that filamentous algae account for the labeled material. It is possible that the label is present in a small amount of protein. Protein has been shown to be associated with some native chitins (Hackman, 1960). The detection of ^{14}C in components of coral matrix may be a consequence of the relatively rapid growth rate of hermatypic corals. The ability to label the matrix may provide a tool for analysis of matrix synthesis and its relation to calcification in *Pocillopora*.

Some observations reported here on corals reveal interesting parallels with those reported for sea anemones by Trench (1969). He showed that algae from anemones and other coelenterates liberate glycerol *in vitro* and that excretion is enhanced by the presence of host tissue homogenate. He also demonstrated that in *in vivo* experiments anemones assimilate about 22–45% of the ^{14}C fixed by algae, mostly into lipid and protein.

Although the results of the present investigation provide evidence for a contribution by zooxanthellae to coral reef organic productivity, they do not demonstrate quantitatively that this represents a phenomenon of selective advantage to the hosts. This can only emerge from quantitative experiments on maintenance, growth and survival of symbiotic and algae-free coral tissues of the same species.

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Summary

1. The hermatypic coral, *Pocillopora damicornis* was incubated in the laboratory and in its reef habitat with $Na_2{}^{14}CO_3$ for 1–24 hours. Controls were incubated in darkness. ^{14}C fixation in light exceeded that in darkness.

2. Fractionation of corals labeled on the reef for 24 hours revealed that 35–50% of the total ^{14}C fixed appeared in the animal tissue lipid (as ^{14}C-glycerol) and protein. From a comparison with dark controls it is concluded that photosynthetic products of zooxanthellae are translocated to host coral tissue. The skeletal organic matrix also acquires ^{14}C.

3. Zooxanthellae isolated from corals and incubated in a homogenate of host coral tissue selectively release glycerol and traces of other organic material including glucose, alanine, and glycolic acid confirming previous observations.

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NOTE ADDED IN PROOF

Mr. Stephen Young in our laboratory has analysed the distribution of ^{14}C in *P. damicornis* skeleton using a modified fractionation technique. He finds 87% as carbonate-^{14}C, 1% in protein and chitin, and 12% in an unidentified lipid. His comparative studies reveal that protein and lipid are the chief constituents of the organic matrices of most of the 14 species (4 Suborders) examined, and that chitin is the chief constiutent only in a few species, including *P. damicornis.*